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Sulfite facilitates LDL lipid oxidation by transition metal ions: A pro-oxidant in wine?

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Abstract **Lipid oxidation in LDL may play a role in atherogenesis. It has been shown that sulfite – a compound in the aqueous fraction of wine – could inhibit free radical (AAPH) mediated oxidation of plasma. Thus, sulfite has been proposed as an antioxidant. In contrast, the aqueous phase of wine has recently been shown to contain not fully identified compounds promoting transition metal ion (Cu^{2+}) initiated LDL oxidation. As transition metal ions can catalyse the auto-oxidation of sulfite, we studied the influence of sulfite on Cu^{2+} initiated LDL oxidation. The results show that sulfite at concentrations found in vivo strongly facilitated LDL oxidation by Cu^{2+}. The LDL-oxidase activity of ceruloplasmin was also stimulated by sulfite. ROS formation by Cu^{2+}/SO_3^{2-} was not inhibited by SOD but by catalase. We propose that formation of Cu^{+}, sulfite radicals ($SO_3^{\cdot-}$) and hydroxyl radicals ($OH^{\cdot}$) is a mechanism by which sulfite could act as a pro-atherogenic agent in presence of transition metal ions.**

Keywords: LDL oxidation; Sulfite; Lipid oxidation; Copper ions

1. Introduction

The oxidative modification of low density lipoprotein (LDL) may play a role in early atherogenesis [1–4]. This has initiated investigations on antioxidants as possible prophylactic/therapeutic antiatherogenic agents. On the other hand, it is also important to identify compounds or reactions which act as pro-oxidants in LDL lipid oxidation [5–7]. From in vitro and in vivo experiments it has been suggested that in red wine the (poly)phenols have the potential to inhibit LDL oxidation, thus diminishing the risk of cardiovascular disease as reviewed by Wollin et al. [8]. In vitro, LDL oxidation by copper ions and the free radical generating compound AAPH is inhibited by phenols like resveratrol extracted from red wine [9–12]. However, a pro-oxidant effect in the water soluble fraction of red wine was found, but the substance(s) were not identified [12]. On the other hand, when this fraction was added prior to the initiation of LDL oxidation an antioxidant effect was observed [12]. Taking into consideration (i) that the aqueous fraction of wine contains relative high concentrations of sulfite and (ii) that sulfite in presence of catalytically active transition metal ions can form sulfite anion radicals [13–15], which in turn may act as initiators/propagators of lipid oxidation one may speculate that sulfite may facilitate LDL oxidation initiated by Cu^{2+}.

In the present study, we examined the influence of sulfite on the oxidation of LDL initiated by copper ions and on the LDL-oxidase activity of ceruloplasmin.

2. Materials and methods

2.1. Materials

Ceruloplasmin (human) was from Calbiochem. Ascorbate was from Merck. α-Tocopherol, bathocuproine disulfonate (BCS), benzenesulfonic acid, 1-octanesulfonic acid sodium salt, glutathione (GSH), catalase (EC 1.11.1.6. bovine liver) and superoxide dismutase (EC 1.15.1.1. bovine erythrocytes) were from Sigma–Aldrich Chemical Corp. 2′,7′-Dichlorodihydrofluorescin diacetate (H_2DCF-DA) was purchased from Molecular Probes and stored (40 mmol/L in dimethylformamide) at −20 °C. Sodium sulfite (Na_2SO_3, Merck) and all other chemicals were of analytical grade.

2.2. Lipoprotein isolation

LDL preparations were isolated by ultracentrifugation as reported previously [16]. The final preparations were dialysed against 150 mmol/L NaCl containing 0.1 mmol/L EDTA or subjected to gel chromatography to get rid of KBr and filter sterilised. Protein was estimated by a modified Lowry method [17] using bovine serum albumin as a standard. All LDL concentrations are given as mg protein/ml.

2.3. LDL oxidation

Prior to LDL oxidation, the lipoprotein was equilibrated in phosphate buffered saline (PBS), pH 7.4, using Sephadex G-25 chromatography (PD-10 columns, Pharmacia). LDL (0.2 mg/ml) was incubated with the respective compound at 37 °C. In general, sulfite was added to the LDL samples and lipid oxidation was started by the addition of Cu^{2+}. Adding sulfite after Cu^{2+} did not change the results observed.

2.4. Ceruloplasmin preparation

Lyophilized ceruloplasmin was dissolved in distilled water and applied to a small Sephadex column (Pharmacia) equilibrated in PBS and the protein fraction was used in the experiments.

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2.5. Estimation of LDL oxidation

LDL oxidation was monitored as the increase in conjugated diene (CD) formation by measuring $A_{234\ nm}$. Samples were measured using a Hitachi UV/VIS 2000 spectrophotometer equipped with a thermostated (37 °C) 6-position cuvette sampler.

Lag time calculation was done according to Esterbauer et al. [18].

TBARS formation in LDL was measured after oxidation and was stopped by the addition of 50 μmol/L EDTA and 10 μmol/L BHT as reported and expressed as malondialdehyde equivalents [19].

Alteration of relative electrophoretic mobility (REM) of LDL was estimated by agarose gel electrophoresis as reported recently [20]. LDL (1 mg/ml PBS) was incubated with 20 μmol/L Cu^{2+} in the absence or presence of 80 μmol/L sulfite at 37 °C. Reactions were stopped with 100 μmol/L EDTA and after addition of sample buffer 10 μg LDL protein per lane was applied.

2.6. Spectroscopy

Bathocuproine-disulfonate (BCS) reaction with free copper ions in the absence or presence of sulfite was done using Cu^{2+}–glycine complexes (molar ratio 1:6) to avoid interactions of free Cu^{2+} with PBS [21]. Spectra were recorded between 600 and 400 nm.

BCS reaction with ceruloplasmin (Cp) in the absence or presence of sulfite was done using 20 μmol Cp-copper/L PBS, sulfite (500 μmol/L) and BCS (100 μmol/L).

2.7. DCF-fluorescence

2′,7′-Dichlorofluorescin (DCFH) was always freshly prepared by alkaline hydrolysis of its diacetate derivate according to the method of Cathcart et al. [22]. For that purpose, 0.5 ml of 1 mmol/L H_2DCF-DA in water was mixed with 2 ml of 0.01 N NaOH. After incubation in the dark for 30 min, the solution was neutralised with 10 ml of 25 mmol/L PBS, pH 7.2. Cu^{2+}, sulfite, superoxide dismutase (SOD), catalase, GSH, ascorbate and α-tocopherol were pipetted into black flat-bottom 96 well plates (Greiner bio-one) to give the respective concentrations mentioned in the figure legend in a volume of 100 μl. After an incubation of 5 min at 37 °C, 100 μl of DCFH was added to obtain a final concentration of 20 μmol/L. Upon reaction with oxidising species, the highly fluorescent compound 2′,7′-dichlorofluorescein (DCF) was formed and light emission was followed in an automated plate fluorescence reader (Wallac) at 37 °C using an excitation wavelength of 485 nm. Emission was recorded at 535 nm every 5 min over a time period of about 100 min.

2.8. Copper release from ceruloplasmin

Cp (40 μmol Cp-copper/L in 0.15 M/L NaCl) was incubated at 37 °C in the absence or presence of 100 μmol/L sulfite for 3 h. Samples ($n = 2$) were filtered through high-flux Biomax™ ultrafiltration membranes (cut-off MW 10.000, Millipore). The filtrate was analyzed for copper released. A standard curve using copper sulfate was used for calculation of unknowns.

2.9. Statistical analysis

Data were calculated as means ± standard deviation (S.D.) of 3–7 experiments. Specific effects were evaluated by one-way analysis of variance (ANOVA) plus Tukey–Kramer multiple comparisons test. $P < 0.05$ was regarded statistically significant.

3. Results

Fig. 1 shows the influence of sulfite (0–20 μmol/L) on LDL oxidation initiated by Cu^{2+} (5 μmol/L) monitored as increase in conjugated diene formation. As low as 1.25 μmol/L sulfite showed a stimulating effect on lipid oxidation. Lag time of LDL oxidation was shortened from 9.8 min (control) to 1.7 min by 20 μmol/L, to 2.8 min by 10 μmol/L, to 4.4 min by 5 μmol/L, to 5.7 by 2.5 μmol/L and to 6.6 min by 1.25 μmol/L sulfite, respectively. The maximal amount of conjugated diene formation was not altered by sulfite (Fig. 1).

Fig. 2 depicts the Cu^{2+} concentration dependent LDL oxidation in absence or presence of 10 μmol/L sulfite. At the lowest Cu^{2+} concentration used (0.625 μmol) sulfite facilitated lipid oxidation by 0.625 μmol/L Cu^{2+} to an extent equivalent to the oxidation seen with 1.24 μmol/L Cu^{2+} in the absence of sulfite. In contrast to inorganic sulfite, "organic" sulfite used as benzolsulfonic acid and octylsulfonic acid showed no facilitating action on Cu^{2+} initiated LDL oxidation (not shown).

Next we tested the influence of sulfite on lipid oxidation when LDL oxidation was already in progress. As seen in Fig. 3 sulfite showed rapid increase in CD formation when added during the propagation phase.

TBARS formation and alteration in REM in Cu^{2+} oxidized LDL was also facilitated in the presence of sulfite (Fig. 4).

The copper binding (transport) protein ceruloplasmin has been found to catalyse lipid oxidation in LDL [23]. LDL-oxidase activity of ceruloplasmin is stimulated by vitamin C or homocysteine by reducing the redox active Cu^{2+} in the protein [24,25]. As seen in Fig. 5 using 0.6 μmol/L ceruloplasmin corresponding to 4.2 μmol/L total copper assuming 7 copper atoms [23] there was no significant increase in lipid oxidation observed over 120 min compared to free copper ions (Fig. 1). In presence of sulfite (10 μmol/L) no facilitation of conjugated diene formation was found up to 120 min in contrast to free Cu^{2+}. However, after prolonged incubation time (2.5 h) sulfite increased CD formation in presence of ceruloplasmin and after 5 h produced about 3-fold more conjugated dienes compared to ceruloplasmin alone (Fig. 5).

Measurements of copper release from Cp revealed that under these conditions no copper is released after 3 h with or without sulfite.

Next, we used a DCF fluorescence assay (see Section 2) to detect ROS in sulfite/Cu^{2+} incubations [26]. As seen in Fig. 6, Cu^{2+} (5 μmol/L) alone had a very limited effect on relative fluorescence increase over time compared to control. However, in presence of sulfite (20 μmol/L) fluorescence development was strongly increased (Fig. 6). Calculations from the linear part of the reaction curve revealed that the addition of sulfite resulted in an 1450 ± 211% ($n = 6$) increase in DCF-fluorescence compared to control (100 ± 27.6%, $n = 7$; $P < 0.001$). Sulfite alone showed 137 ± 37.2% ($n = 6$; n.s.) and Cu^{2+} alone 191 ± 21.5% ($n = 6$; n.s.) increase, respectively. The inset in Fig. 6 shows that the sulfite effect on Cu^{2+} induced DCF oxidation flattens between 50 and 100 μmol/L sulfite.

To evaluate the effect of antioxidants on the observed sulfite effect, vitamins C and E were tested. As seen in Fig. 7, both vitamins tested up to 100 μmol/L did not inhibit the action of sulfite.

Superoxide dismutase and catalase have been supposed as an useful tool to study radical reactions [27]. Fig. 8 depicts the influence of SOD, catalase and GSH on the sulfite/Cu^{2+} promoted DCF oxidation. SOD had virtually no effect (96,2 ± 2.1% compared to control 100 ± 2.80%) whereas catalase inhibited DCF oxidation (67.4 ± 6.1% versus control) and GSH almost completely prevented oxidation by sulfite/Cu^{2+} (4.1 ± 1.5% versus control). A small additive inhibitory effect of SOD/catalase was observed (59.6 ± 3.5% compared to control).

In the Cu^{2+}/sulfite initiated LDL oxidation system, catalase at 200 U/ml showed a strong inhibitory effect on conjugated diene formation, whereas SOD was much less effective (Fig. 9).

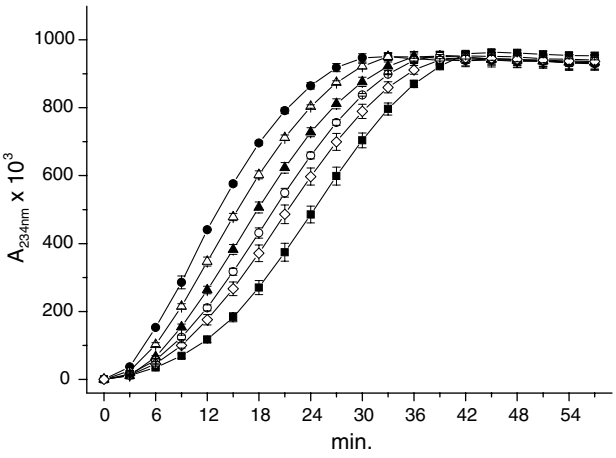

Fig. 1. Influence of sulfite on Cu^{2+} initiated LDL oxidation. LDL (0.2 mg/ml PBS) was incubated with 5 μmol/L Cu^{2+} (■) in the absence or presence of sulfite (1.25 up to 20 μmol/L: ◇ = 1.25 μmol/L, ○ = 2.5 μmol/L, ▲ = 5 μmol/L, △ = 10 μmol/L, ● = 20 μmol/L). LDL oxidation was monitored by conjugated diene formation measured as the increase in absorbance at 234 nm ($n = 3$).

From a redox-chemical point of view one may assume that the sulfite/Cu^{2+} reaction may result in the formation of Cu^{+}. Therefore, Cu^{2+} was incubated in the absence or presence of sulfite and the Cu^{+}-reagent bathocuproine-disulfonate (BCS) and the spectra were recorded between 600 and 400 nm. As seen in Fig. 6 the addition of sulfite (1 mmol/L PBS) to Cu^{2+} (50 μmol/L PBS) resulted in the formation of the Cu^{+}–BCS complex with its absorption maximum at 480 nm (Fig. 10). The inset in Fig. 10 shows that sulfite was also able to induce Cu^{+} formation in ceruloplasmin.

4. Discussion

Sulfur dioxide, metabisulfite, bisulfite and sulfite (sulfiting agents) are widely used as preservatives and sulfite enters the body in foods, beverages and drugs [28]. Endogenous sulfite is generated during the normal processing of sulfur-containing amino acids [29]. Sulfite in wine can reach up to 6 mmol/L and the consumption of a glass of wine results in a plasma sulfite concentration of about 10 μmol/L [30]. Using AAPH as a free radical generating compound it had been shown that sulfite could inhibit plasma lipid oxidation as monitored by malondialdehyde formation [30]. From these results it was concluded that sulfite may act as antiatherogenic compound in wine. On the other hand, recently it has been reported by Hötzer et al. that the aqueous fraction of red wine contains compounds which could promote Cu^{2+} initiated oxidation of LDL [12]. However, an inhibitory action was observed when the fraction was added prior to the initiation of oxidation. As sulfite in presence of transition metal ions can undergo free radical formation which can attack biomolecules like nucleic acids, lipids and enzymes [13–15,31,32] one may assume that Cu^{2+} initiated LDL oxidation may be stimulated by sulfite. Therefore, in the present paper the influence of sulfite on LDL oxidation initiated by free copper ions or ceruloplasmin was investigated. The results showed that sulfite at very low concentrations which can easily be reached in vivo after the consumption of wine could promote lipid oxidation by copper

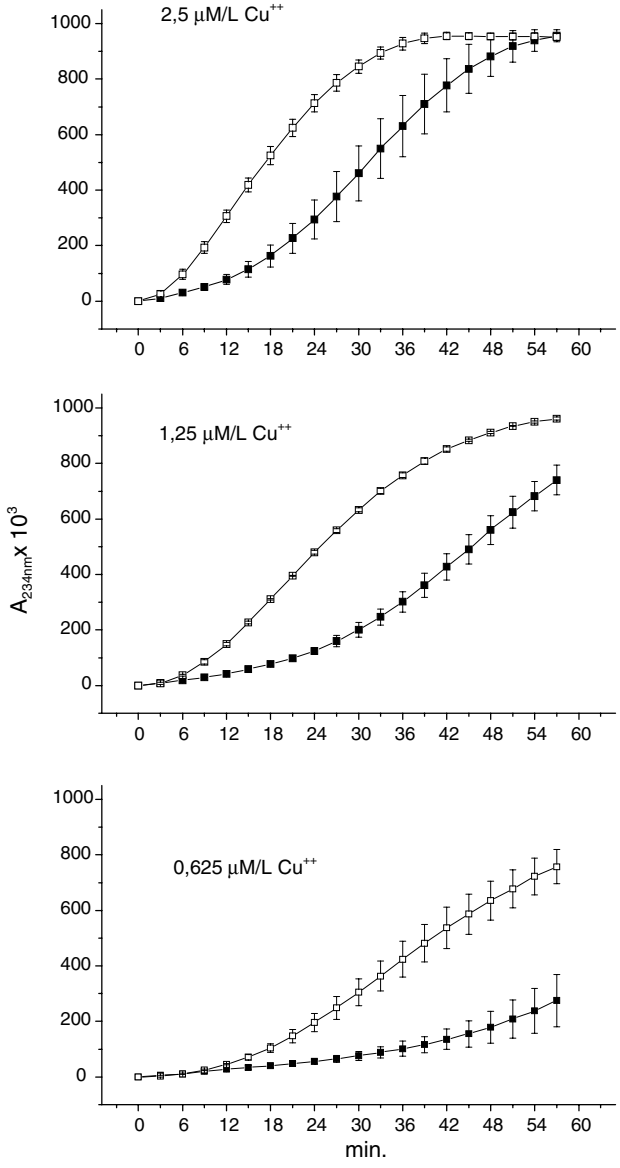

Fig. 2. Influence of Cu^{2+} concentration on Cu/sulfite initiated LDL oxidation. LDL (0.2 mg/ml PBS) was incubated with 10 μmol/L sulfite (□) in presence of Cu^{2+} (■, 0.625 up to 2.5 μmol/L). LDL oxidation was monitored by conjugated diene formation measured as the increase in absorbance at 234 nm ($n = 3$).

ions in LDL. The stimulatory effect of sulfite was seen when sulfite was added at the beginning of the oxidation reaction and when added during the propagation phase of LDL oxidation in our experiments. This is a noticeable difference to the observations of Hötzer et al. [12]. As these authors used total red wine extracts, one may speculate that the interaction of sulfite with other substances may be responsible for the observed differences. It is still a matter of debate if transition metal ions are involved in LDL oxidation (atherogenesis) in vivo. Redox active metal ions have been found in atherosclerotic plaques by Smith et al. [33] and very recently Stadler et al. [34] reported on detection and quantification of transition metal ions in human atherosclerotic plaques and found evidence of elevated levels of copper and iron. Thus these metal ions may contribute to the progression of the disease.

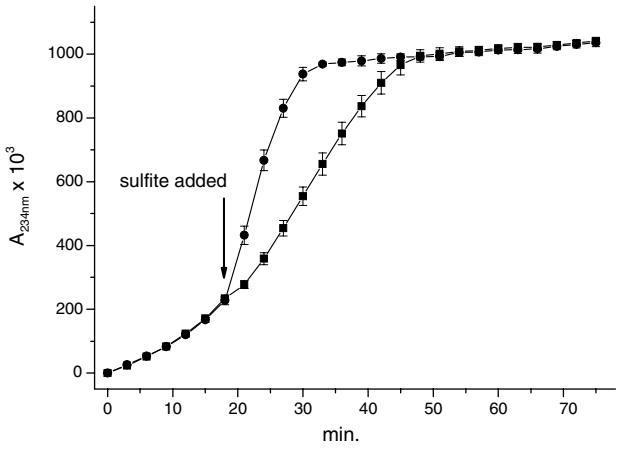

Fig. 3. Influence of sulfite added during the propagation phase on Cu-$^{2+}$ initiated LDL oxidation. LDL (0.2 mg/ml PBS) was incubated with 5 μmol/L Cu^{2+} (■). After 18 min of oxidation (see arrow), sulfite (●, 20 μmol/L) was added. LDL oxidation was monitored by conjugated diene formation measured as the increase in absorbance at 234 nm ($n = 3$).

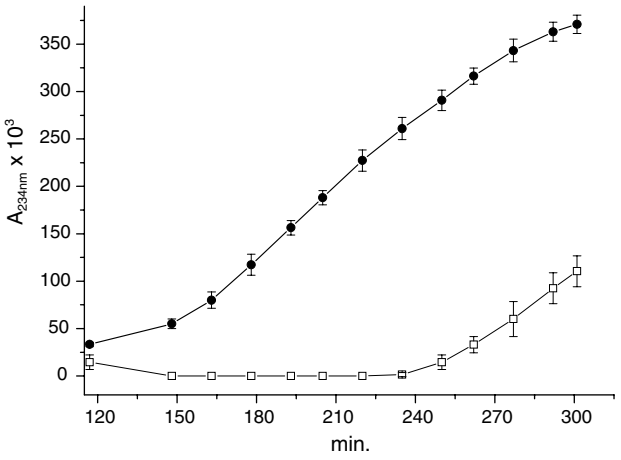

Fig. 5. Influence of sulfite on the LDL-oxidase activity of ceruloplasmin. LDL (0.2 mg/ml PBS) was incubated with 0.6 μmol/L ceruloplasmin in the absence (□) or presence (●) of 10 μmol/L sulfite. LDL oxidation was monitored by conjugated diene formation measured as the increase in absorbance at 234 nm. ($n = 3$).

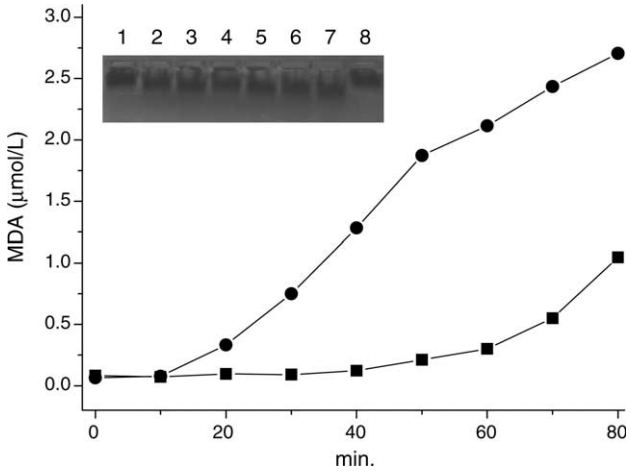

Fig. 4. Influence of sulfite on Cu^{2+} initiated LDL oxidation. LDL (0.2 mg/ml PBS) was incubated with 5 μmol/L Cu^{2+} in the absence (■) or presence (●) of sulfite (20 μmol/L). LDL oxidation was monitored by TBARS formation and expressed as malondialdehyde equivalents (one representative experiment out of three is depicted). Inset shows the alteration in REM after incubation for 20, 40 and 60 min at 37 °C with the respective compounds as analyzed by agarose gel electrophoresis (see Section 2). 1 and 8: LDL, 2: 20 min. LDL + Cu^{2+}, 3: 20 min. LDL + Cu^{2+} + sulfite, 4: 40 min. LDL + Cu^{2+}, 5: 40 min. LDL + Cu^{2+}+sulfite, 6: 60 min. LDL + Cu^{2+}, 7: 60 min. LDL+Cu^{2+} + sulfite.

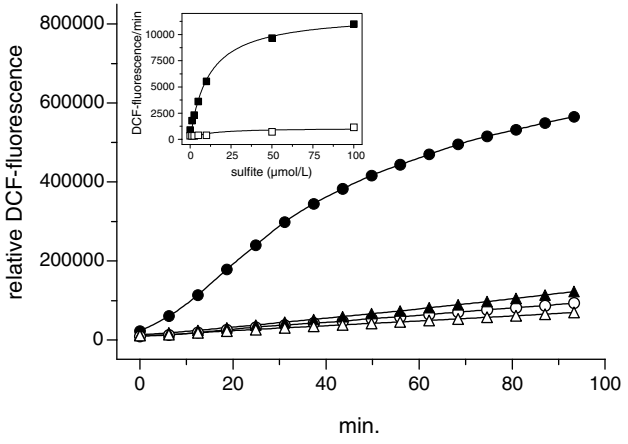

Fig. 6. Measurement of ROS by DCF-fluorescence. 100 μl of 40 μmol/L DCFH was added to 100 μl of PBS (△) containing 20 μmol/L sulfite (○), 5 μmol/L Cu^{2+} (▲) or 5 μmol/L Cu^{2+} together with 20 μmol/L sulfite (●) and the fluorescence intensity of DCF was followed with excitation/emission wavelengths set at 485/535 nm. The inset shows the oxidation of DCFH due to increasing concentrations of sulfite in the absence (□) and presence of 5 μmol/L Cu^{2+} (■). Results are means of five replicates of one experiment representative of six experiments performed. The rate of oxidation was calculated from the linear initial slopes from time-course data (<45 min).

In contrast to free Cu^{2+}, Cu^{2+} bound to ceruloplasmin was not as effective in initiating LDL oxidation. Sulfite stimulated the LDL oxidase activity of ceruloplasmin, but only after prolonged incubation whereas LDL oxidation in presence of free Cu^{2+} was immediately stimulated by sulfite. It should be mentioned that some authors have raised critical points regarding Cp mediated lipid oxidation due to the labile nature of this plasma protein [35,36].

LDL oxidase activity of Cp is stimulated by the formation of Cu^{+} from redox active Cu^{2+} bound to the protein [23–25]. Using the bathocuproine disulfonate complex-reaction to detect the formation of Cu^{+}, the results show that in the presence of sulfite free and Cp-bound Cu^{2+} is rapidly reduced to Cu^{+}. As sulfite did not cause copper release from Cp, this may be the reason for the sulfite-increased LDL oxidase activity of Cp. Cu^{+} by redox cycling is further involved in lipid oxidation facilitating the decomposition of lipid hydroperoxides to chaincarrying radicals [37].

It has been reported that SO_3^{2-} can undergo an one-electron oxidation forming sulfur trioxide anion radical $SO_3^{\bullet-}$ catalysed by transition metal ions or peroxidases [15]. Thus, formation of both Cu^{+} and the sulfite radical anion could be responsible for the facilitating effect of sulfite observed on Cu^{2+} mediated LDL oxidation. In the DCF fluorescence assay which detects

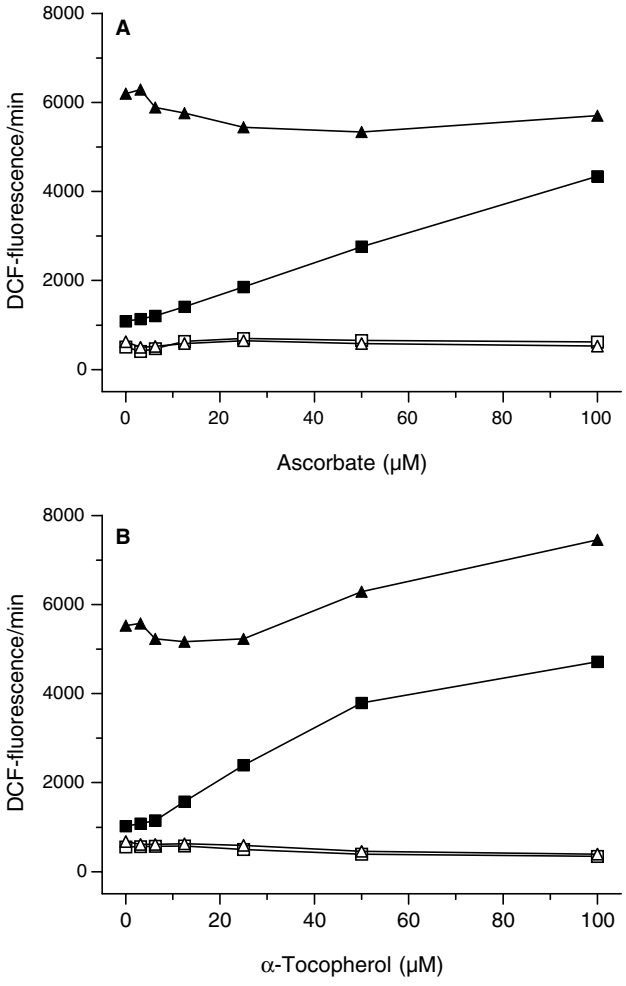

Fig. 7. Influence of vitamin C and vitamin E on Cu^{2+}/sulfite mediated DCF-fluorescence. PBS (□) with 20 μmol/L sulfite (△), 5 μmol/L Cu^{2+} (■) and 5 μmol/L Cu^{2+} together with 20 μmol/L sulfite (▲) was incubated with various concentrations of ascorbate (A) and α-tocopherol (B). α-Tocopherol was dissolved at 25 mmol/L in ethanol. Controls with the solvent showed no effect. The graphs show one representative experiment of three performed.

ROS, we could show that Cu^{2+} in presence of sulfite led to the formation of significant amounts of oxidant species. This was not inhibited by ascorbate and α-tocopherol. Both vitamins have the potential to reduce Cu^{2+} to Cu^{+} a reaction which may be the reason for the inability of the compounds to act as antioxidants in this system. Using SOD and catalase to study the radical/ROS involved in the sulfite/Cu^{2+} reaction the results show that catalase could inhibit ROS formation in contrast to SOD indicating that H_2O_2 is formed and not the superoxide radical $O_2^{\bullet-}$ in presence of sulfite and Cu^{2+}. H_2O_2 alone is a poor LDL oxidising agent, but H_2O_2 in presence of Cu^{+} can undergo the copper-Fenton reaction ($Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + OH^{\bullet} + OH^{-}$) resulting in the formation of the hydroxyl radical, a strong lipid oxidising agent [37]. Catalase was also more powerful in inhibiting LDL oxidation by Cu^{2+} in absence or presence of sulfite compared to the action of SOD.

Thus beside the formation of $SO_3^{\bullet-}$ and Cu^{+} the formation of $OH^{\bullet}$ may play a role in LDL oxidation in presence of sulfite/Cu^{2+}.

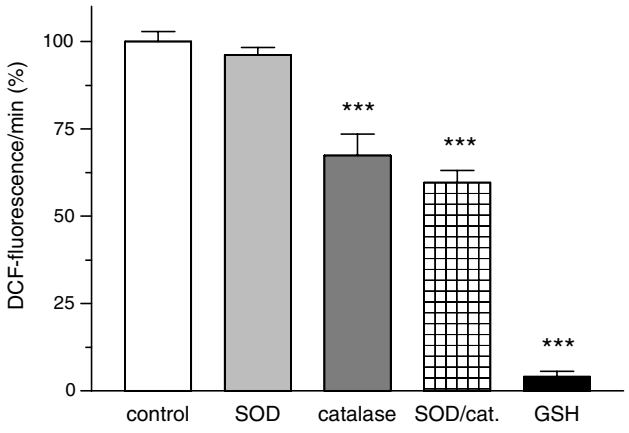

Fig. 8. Influence of SOD, catalase and GSH on Cu^{2+}/sulfite mediated ROS formation. 5 μmol/L Cu^{2+} and 20 μmol/L sulfite were incubated with 20 U SOD, 20 U catalase, both enzymes together or 1 mmol/L GSH at 37 °C for 5 min and DCFH was added. The slopes of the linear DCF-formation of the time-course data (i.e., <45 min) were calculated and compared with the values from Cu^{2+}/sulfite (open bar). Results are means ± S.D. for three experiments with $^{***}P < 0.001$ versus control.

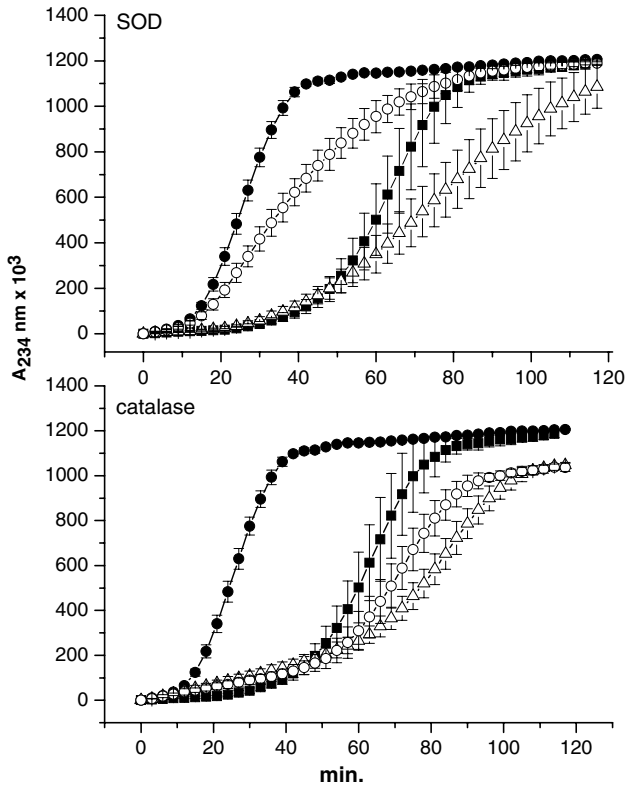

Fig. 9. Effect of SOD and catalase on Cu^{2+} and Cu^{2+}/sulfite initiated LDL oxidation. LDL (0.2 mg/ml PBS) was incubated with 5 μmol/L Cu^{2+} in the absence or presence of sulfite (20 μmol/L). SOD (200 U/ml) or catalase (200 U/ml) were added to the respective incubations. ■, Cu^{2+}; ●, Cu^{2+}/sulfite; △, Cu^{2+}/SOD (upper panel) or Cu^{2+}/catalase (lower panel); ○, Cu^{2+}/sulfite/SOD (upper panel) or Cu^{2+}/sulfite/catalase (lower panel). LDL oxidation was monitored by conjugated diene formation measured as the increase in absorbance at 234 nm ($n = 3$).

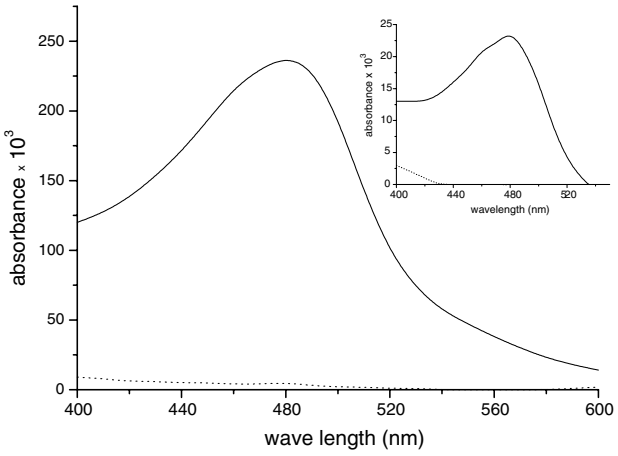

Fig. 10. Sulfite mediated Cu^{+} formation. Cu^{2+} (50 μmol/L PBS) was incubated with 100 μmol/L BCS in the absence (···) or presence (—) of 1 mmol/L sulfite and after 30 s the spectra were recorded between 600 and 400 nm to monitor the Cu^{+}–BCS complex. Inset: 20 μmol/L Cp-copper in PBS was incubated with 100 μmol/L BCS in the absence (···) or presence (—) of 500 μmol/L sulfite and after 30 min spectra were recorded.

Taken together the results indicate that sulfite, which can reach abundant concentrations in vivo, can react as an anti- or pro-oxidant of LDL lipid oxidation depending on the nature of the oxidant insult (free radical or transition metal ion) involved. Thus, inorganic sulfite behaves as a "Janus-faced" molecule – a property which has been attributed to some anti-oxidative compounds [38].

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